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Pharmacological disruption of the MID1/α4 interaction reduces mutant Huntingtin levels in primary neuronal cultures

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Abstract

Expression of mutant Huntingtin (HTT) protein is central to the pathophysiology of Huntington's Disease (HD). The E3 ubiquitin ligase MID1 appears to have a key role in facilitating translation of the mutant HTT mRNA suggesting that interference with the function of this complex could be an attractive therapeutic approach. Here we describe a peptide that is able to disrupt the interaction between MID1 and the α4 protein, a regulatory subunit of protein phosphatase 2A (PP2A). By fusing this peptide to a sequence from the HIV-TAT protein we demonstrate that the peptide can disrupt the interaction within cells and show that this results in a decrease in levels of ribosomal S6 phosphorylation and HTT expression in cultures of cerebellar granule neurones derived from $Hdh^{Q111/Q7}$ mice. This data serves to validate this pathway and paves the way for the discovery of small molecule inhibitors of this interaction as potential therapies for HD.

Keywords: Huntington's Disease; MID1; cerebellar granule cell culture

Abbreviations:

HTT	Huntingtin
HD	Huntington's Disease
MID1	Midline 1
PP2A	Protein Phosphatase 2A
mHTT	Mutant Huntingtin
wtHTT	Wild type Huntingtin
S6K	40S ribosomal S6 kinase
mTOR	Mammalian target of rapamycin
CGN	Cerebellar granule neurons
MAP2	Microtubule Associated Protein 2
GFAP	Glial Fibrillary Protein
aCSF	Artificial cerebral spinal fluid
DIV	Days in vitro

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder affecting around 5-10 individuals *per* 100,000. Patients show motor dysfunction, psychiatric impairment and progressive dementia. The only currently available therapies provide limited symptomatic treatments of the emotional disturbance or chorea but do nothing to change the progressive nature of the condition [1-3] so disease-modifying approaches to treat this devastating disease are urgently needed.

HD is caused by mutations in the Huntingtin (HTT) gene consisting of expansions of the poly-CAG repeat in exon 1 of *HTT* [4] resulting in production of mutant HTT (mHTT) protein containing an extended glutamine tract in the N-terminal domain. Expression of the mutant protein appears to have a wide range of detrimental effects such as transcriptional dysregulation, mitochondrial dysfunction, altered vesicle recycling, increased autophagy and impaired axonal transport [5], which together result in neuronal dysfunction and eventually cell death [6-11]**.** While the mHTT mRNA may contribute to the observed toxicity [3, 12-15], the majority of evidence suggests that the mHTT protein is the primary driver of disease pathology**.**

A promising therapeutic approach is to lower mHTT expression by using oligonucleotides [16, 17]. Administration of antisense oligonucleotides targeting HTT into the cerebral spinal fluid of transgenic mice resulted in reduced levels of HTT within the brain [17] and the long-term rescue of disease symptoms, providing hope that at least some of the manifestations of disease could be reversible.

We recently identified a novel mechanism where translation of mHTT mRNA is regulated by the MID1-PP2A protein complex [18]. Phosphorylation of 40S ribosomal S6 kinase (S6K) is a key step in initiating protein translation and this is regulated by the balance between phosphorylation (carried out by mammalian target of rapamycin mTOR kinase (mTOR)) and dephosphorylation (carried out by protein phosphatase 2A (PP2A)). MID1 is a microtubule-

associated E3 ubiquitin ligase, which associates with α4 (IGBP1), a regulatory subunit of PP2A [19], and targets PP2A for proteasomal degradation [19]. This results in increased phosphorylation of S6 kinase and thereby facilitation of translation of those mRNAs, which are associated with the complex [18-20]. mHTT mRNA associates with the MID1 protein complex in a CAG-repeat length dependent manner, thereby resulting in increased production of mHTT protein [18]. Based on this finding, we hypothesize that disruption of the MID1 complex would lead to an inhibition of the synthesis of aberrant HTT protein and set out to explore the potential of the MID1 complex as a therapeutic target for HD.

Materials and Methods

Binding of peptides to MID1

Constructs to express N-terminal Flag and glutathione S-transferase dual tagged versions of human MID1-Bbox1, residues 110-165 and human MID1-Bbox1-Bbox2, residues 110-214 were synthesised at GenScript, China and subcloned into pET24b (Merck). Details of construct production are found in supplementary information.

Peptides containing a 29-residue sequence from α4 (AQAKVFGAGYPSLPTMTVSDWYEQHRKYG) were synthesized using standard peptide synthesis procedures and HPLC-purified to >95% purity by Cambridge Research Biochemicals (Cambridge, UK). This peptide was labelled with Alexa647 on the N-terminus through the addition of a Cys residue and maleimide dye coupling (GSK'454A) and was used to set up binding assays. A peptide containing a point mutation (Y721A) was also synthesised (AQAKVFGAGYPSLPTMTVSDWAEQHRKYG) and both sequences were tagged with an N-terminal sequence derived from HIV-TAT protein (RKKRRQRRR) to yield GSK'364A and GSK'365A.

Association of the MID1 proteins with GSK'454A was measured by monitoring the fluorescence polarisation (FP) of peptide upon titration of MID1 proteins. Details of the conditions used for binding studies are found in supplementary information.

To demonstrate specific binding of Alexa647-labelled peptide to the MID1 proteins competition experiments were prepared by titrating non-labelled α4-peptide (GSK'037A) or TAT-tagged α4-peptide (GSK'364A, GSK365A) into pre-formed MID1: GSK'454A complex. The FP data was plotted as a function of unlabelled α4 peptide concentration and fitted to a 4-parameter logistic equation to determine IC50's.

Disruption of α4/MID1 binding in cells

The interaction between MID1 and α4 protein within living cells was monitored using a NanoBRET™ assay, which was established by Promega. NanoBRET™ is a proximity-based assay dependent upon energy transfer from a luminescent donor to a fluorescent acceptor. Here, Nanoluciferase (NanoLuc) protein was fused to the C-terminus of full-length α4 (donor component), and the HaloTag® protein was fused to the N-terminus of full-length MID1 (acceptor component).

The NanoBRET response was measured in the presence of NanoLuc substrate (50 µM FAC) on the PerkinElmer multimodal Envision plate reader (equipped with 450 nm band-pass and 610 nm long-pass filters). The response was reported as a NanoBRET™ ratio of acceptor/donor and converted to milliBRET units (mBU; whole numbers) using the following equation: mBU = $(610_{EM}/450_{EM})*1000$. To account for donor-contributed background or bleed-through, the DMSO plate values (no acceptor) were subtracted from the NanoBRET™ 618 Ligand plate values (with acceptor). Data were fitted to a 4-parameter logistic equation to determine IC50.

Optimisation of cell culture conditions

A complete protocol of the preparation and characterisation of primary cerebellar granule neuron (CGN) cultures is found in supplementary info. Twenty–four hours after seeding, a complete change of medium containing 2 µM cytosine arabinoside (Sigma C1768) was performed. To study the turnover of HTT protein in culture, cell culture medium was either left unchanged for 18 days to deprive cells of fresh nutrients, or half of the volume was changed every 2-3 days to replenish nutrients. Glial-conditioned media was also used to determine the importance of glial support for HTT expression in CGNs (supplementary information). Long-term expression of HTT *in vitro* was characterised by western blotting (detailed below). In addition, Ca^{2+} imaging was carried out to investigate the functionality of neurons in culture without media change.

The effect of peptides on HTT levels on primary CGN

Primary CGNs (day *in vitro* 1, DIV1) were incubated in culture medium containing 1, 3 or 30 µM GSK'364A or GSK'365A made up in 0.4% DMSO for 24 hr. As controls, CGNs were incubated in culture medium with 0.4% DMSO. At the end of treatment, the 4 samples per treatment were harvested for cell viability assay, immunohistochemistry, or Western blotting analysis.

Detection of changes in neuronal morphology

Details of the immunohistochemistry protocol are found in supplementary data. DAPI was used to label cell nuclei and Phalloidin-Atto488 was also used to detect actin in CGNs and to visualise neuronal morphology. Fluorescent images were acquired using a confocal laser

scanning microscope (Leica SP5) with a x40 oil immersion objective (NA 1.4) as soon as the mounting medium has cured.

Identification of HTT protein and changes in protein expression after GSK'364A treatment

CGN lysates were harvested with lysis buffer containing 15 mM Tris-HCl (pH 7.5), 48% urea, 8.7% glycerol, 1% SDS, 143 mM mercaptoethanol and 0.004% bromphenol blue. Brain lysates from $Hdh^{Q111/Q111}$ mice were used as positive controls for HTT protein detection. Lysates were heated at 95^{o}C for 5 min and then centrifuged at 14,800 rpm for 5 min. Proteins were separated on a large format electrophoresis system PROTEAN® II xi (BioRad) and immobilised onto PVDF membranes (IPVH00010, Millipore). Primary antibodies against HTT, GAPDH, pS6, S6, GFAP and MAP2 were used to detect expression of these proteins in CGNs after GSK'364 treatment. Details of antibody origin and concentration can be found in supplementary data. Corresponding secondary HRP-tagged antibodies were used to probe for primary antibody binding. The protein signals were detected using an ECL Western blotting analysis system (RPN2232, GE Healthcare Life Sciences) and Fuji Medical X-ray films.

Functional analysis of cultured primary CGN

At the end of culture, Ca^{2+} imaging on primary CGNs was carried out to investigate responsiveness of neurons in culture. This was achieved by incubation in 4.5 µM Fura-2, AM (F1221, ThermoFisher Scientific) in high Mg^{2+} aCSF (in mM: 1.2 $MgCl_2$, 120 NaCl, 3.5 KCl, 0.4 KH_2PO_4, 5 $NaHCO_3$, 1.2 Na_2SO_4, 10 glucose, 1 $CaCl_2$ and 20 HEPES, pH 7.4) for 45 min. Ca^{2+} imaging experiments were then carried out in low Mg^{2+} (0.1 mM) aCSF. Excitability of neurons was recorded after 30, 100 and 300 µM NMDA stimulation followed

by 40 mM KCl stimulation. Images were acquired via a Zeiss Axiovert 200 microscope equipped with a 40X objective controlled by Metafluor Fluorescence Ratio Imaging Software (Molecular Devices). Ca^{2+}-free Fura-2 AM was excited by a UV lamp at 340 nm and at 380 nm when bound to Ca^{2+}. Fluorescent emissions were collected by a FITC filter. The change in 340/380 ratio was detected by MetaFluor data recording software.

Results

Peptides derived from α4 bind to MID1 BBoxes.

MID1 is a microtubule-associated E3 ubiquitin ligase with a number of well-characterised functional domains. The interaction with α4 is mediated by the Bbox domains (Figure 1A). Addition of recombinant MID1/BBox proteins to an Alexa647 labelled α4-derived peptide (GSK'447) resulted in a dose dependent increase in fluorescence polarisation (FP) [21], indicating binding of the peptide to the protein (Figure 1B). The data was fitted to a single-site binding model yielding mean apparent K_d values of 0.6 µM (n=3) for BBox 1 and 16µM (n=3) for BBox 1/2 (supplementary table 1). This binding could be fully inhibited in the presence of a peptide of the same sequence but lacking the Alexa647 label (Figure 1C), yielding a mean IC_{50} of 5.0 µM (n=13) for the BBox 1 construct and 2.5 µM (n=2) for the BBox1/2 construct (supplementary table 1). This competition was specific as a peptide with a single amino acid substitution (Y271A) did not show inhibitory activity (Supplementary Figure 1).

Peptide inhibitors with a cell-entry signal disrupt the MID1/α4 complex in a cellular context.

To allow the activity of the α4 peptide to be explored in a cellular context, peptides containing an HIV TAT-sequence prior to the α4 sequence were generated corresponding to the active peptide (GSK'364A) and the Y271A mutant (GSK'365). GSK'364 inhibited the binding of the fluorescently labelled peptide to MID1 BBox1 with a mean IC_{50} of 1 µM (n=4), while GSK'365 was inactive (Figure 1C).

To monitor the interaction between MID1 and α4 in a cellular context, an in-cell interaction assay using full length proteins was established using Nano-BRET technology (Promega) [22]. Expression of both proteins together in HEK293 cells generated a BRET signal indicating that the proteins were interacting. Addition of GSK'364 to the cells reduced this

signal in a dose dependent manner with a mean IC_{50} of 65 ± 2 µM (n=3) while GSK'365 had no effect (Figure 1D).

Cerebellar granule neurons in culture lose expression of HTT.

Cerebellar granule neurons were isolated from $Hdh^{Q111/Q7}$ animals and maintained in culture. The cells remained viable in culture for at least two weeks and at DIV16 they were challenged with increasing concentrations of NMDA to determine if the cultures were functionally active. As shown in Figure 2A, 30, 100 and 300 µM NMDA elicited a dose-response increase in Fura-2 fluorescence which reached a similar magnitude to that generated by 40 mM KCl, suggesting a maximum increase in intracellular Ca^{2+} concentration was achieved.

HTT and pS6 expression in CGNs cultured up to 18 days in vitro (DIV) were examined. Although CGNs remain functional at DIV16, expression of mHTT and wtHTT protein decreased during the first week in culture such that by day 9 mHTT and wtHTT was hardly detectable (Figure 2C, n=3 per timepoint). The housekeeping protein GAPDH did not significantly change in expression throughout the culture period. Interestingly, the loss of HTT was accompanied by a decrease in pS6 levels, suggesting that translation rates may be decreased in these cells.

Nutrient replenishment is necessary to maintain HTT expression in culture.

Ruling out support from glial cells as a hypothesis for neurons to maintain HTT expression (Supplementary Figure 2), we tested the effects of nutrient replenishment on HTT expression in CGNs since pS6 can be a marker for nutrient deprivation. When half the

volume of culture media was replaced by fresh media every 2-3 days, a steady long-term expression of HTT in the cultured neurons was achieved (Figure 3A). Moreover pS6 levels were also maintained. Interestingly, pS6 levels spiked at DIV17 and 21, which coincided with recent media replenishment. Phalloidin staining of neuronal culture showed good neuronal morphology and formation of neuronal projections and connections at DIV15 (Figure 3B). These results suggested that HTT levels in cultured neurons have a rapid turnover and require maintained S6 protein phosphorylation for the synthesis of HTT protein.

Effect of GSK'364A on S6 phosphorylation and the expression of HTT.

The decline in HTT levels seen in the absence of nutrient replenishment suggested that turnover of HTT protein in cerebellar granule neurons in culture was rapid. Cell cultures were therefore treated with GSK'364A or GSK'365 for 24hrs and protein expression assessed by Western blotting analysis. Treatment with either peptide at a concentration of 30 µM resulted in a significant decrease in levels of both mHTT and wtHTT (Figure 4Ai and ii). Treatment with GSK365 at the same concentration had a smaller impact on expression levels, although the decrease still significant for the mutant protein. This concentration of peptide had no effect on levels of the glial cell marker GFAP, the neuronal marker MAP2 or the housekeeping protein GAPDH (Figure 4Ai). There was no apparent effect on cell viability as assessed by ethidium bromide staining compared to DMSO treatment (Supplementary Figure 3). In addition, MAP2 and GFAP labelling revealed no difference in cell morphology between control and α4 peptide treated cell cultures (Supplementary Figure 3).

No effect on HTT protein levels was observed following treatment of the cells with GSK'364 at 1 or 3 µM (Supplementary Figure 4A and Bi).

If the inhibition of HTT production is due to an effect on the MID1 protein complex, we might expect to see a concomitant effect of the peptide on phosphorylation of S6 and potentially an increase in microtubule-associated PP2A levels. At 30 µM GSK'364A and GSK365A

significantly reduced levels of S6 phosphorylation (Figure 4Aiii), while lower concentrations had no effect (Supplementary Figure 4). Total PP2A levels did not alter significantly after treatment with peptide (Figure 4B).

Discussion

HD is a devastating condition and finding a way to modify the inexorable decline in cognitive and motor function in sufferers would be a major therapeutic breakthrough. The reported success of the antisense approach in the transgenic models suggests that this could be achieved by modulating expression of HTT, and moreover that intermittent knock down could provide a long-term benefit. This approach is reportedly heading into clinical development, but does require administration directly into the brain to achieve efficacy. Alternative targets that might achieve the same goal while being amenable to small molecule drug discovery would be attractive therapeutic opportunities. In this study we demonstrate that pharmacological manipulation of the MID1 complex alters HTT expression in cells and could represent just such a target for future drug discovery efforts.

Here we have shown that a peptide derived from the α4 protein sequence binds to constructs containing the MID1 BBoxes. Based on apparent K_d values the interaction with MID1 BBox1 appears stronger than between MID1 BBox1/2 (Figure 1A), while competition experiments with the unlabelled peptide suggest similar potencies (Figure 1B). It may be that only a fraction of the MID1 BBox1/2 protein is competent to bind the peptide, confounding the direct assessment of affinity. MID1Using the NanoBret technology we were able to establish an assay that assessed formation of the complex between full-length MID1 and a4 proteins within a cellular environment. A version of the α4 peptide containing a cell entry sequence from HIV-TAT (GSK'364A) disrupted this interaction but showed a much lower potency that seen in cell free systems. This likely reflects the challenges of getting high amounts of peptide into the cell, but may also result from the use of full-length protein constructs in the cell environment compared to fragments in the cell free system.

We were able to maintain viable CGN cells in culture for extended periods, but initially observed rapid decline in levels of phosphorylated S6 protein and loss of HTT expression suggesting that ongoing protein production is required to maintain HTT levels within the cells. Indeed previous studies employing RNA interference and antisense oligonucleotides had suggested half-life time of HTT of 24 hours in HEK cells and in mouse striatal neurons [8, 23, 24]. Levels of HTT and phosphorylated S6 levels could be maintained over time by regular replenishment of the media in the culture, suggesting that the mTOR pathway, responsible for nutrient sensing [25], could be involved. In our study, treatment with GSK'364A and GSK365A also resulted in a rapid decline in both proteins consistent with the mode of action being inhibition of HTT translation. This further supports the idea that interfering with HTT translation is a good way to effectively decrease protein levels over a reasonable timeframe.

While less effective than GSK'364, the activity observed in CGN cultures with GSK'365 is unexpected, as this peptide showed minimal activity against MID1 *in vitro* or in the Nano-BRET assay and was intended as a negative control. These data could imply that the observed reduction of HTT levels or pS6 is not related to activity at MID1. However the relative specificity of the effects observed seem to make a general non-specific effect less plausible. We have not been able to test whether this peptide binds to the murine MID1 protein, and it seems more likely that the activity of this point mutant could be species specific. Further work will be required to provide definitive proof of the mechanism of action.

Our previous studies had indicated that the mutant HTT mRNA associated more strongly with the MID1 complex than wtHTT and suggested that inhibition of the complex might have a specific effect on the mutant protein rather than the wild-type protein. However, in these studies GSK'364A affected both wild-type and mutant protein in the same way. The effect is nonetheless relatively specific as control proteins (GAPDH, MAP2, and GFAP) were not affected. The reasons for the apparent discrepancy with the previous study are not clear. However we have obtained preliminary data showing that wtHTT protein levels are reduced in fibroblasts derived from patients with Opitz syndrome, who have mutated MID1 compared

to controls (supplementary Figure 5), suggesting that this pathway may not be as specific for the mutant protein as previously reported. Studies with the peptides in iPS-derived neuronal cells from HD patients would help to determine whether there was any selectivity between normal and mutant proteins in the key target cell.

While it is possible that the peptide could be acting by a number of mechanisms (such as enhancement of autophagy), the effects observed in CGN cultures at 30 μM are consistent with the potency observed in the in-cell interaction assay suggesting that GSK'364A disrupted the MID1-α4 interaction leading to the downstream reduction of S6 phosphorylation and translation of HTT. Others are now reporting similar inhibition of translation of other proteins associated with the complex using GSK'364 and have shown that mRNA levels of these proteins are unaffected [26]. Although we have not confirmed the lack of effect on mRNA levels with the peptide here, we have shown that mutant MID1 causes a reduction in HTT protein levels without affecting levels of mRNA in cells from a patient with Opitz syndrome (supplementary figure 5). As the effect of MID1 on pS6 is thought to depend on degradation of PP2A, inhibition of the complex by GSK'364A might have been expected to result in an increase in PP2A level, which was not observed. However, it may be that the microtubule-associated fraction of PP2A associated with the MID1 complex at any time is only a small proportion of total PP2A, and therefore any changes resulting from disruption of the complex would not be readily detected.

In summary, the data presented in this paper provide strong evidence that the disruption of MID1 protein complex in neurons results in decreased levels of mHTT protein, suggesting that this complex could represent an interesting target for small molecule drug discovery.

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References:

1. Foroud, T., et al., *Differences in duration of Huntington's disease based on age at onset.* J Neurol Neurosurg Psychiatry, 1999. 66(1): p. 52-6.
2. Labbadia, J. and R.I. Morimoto, *Huntington's disease: underlying molecular mechanisms and emerging concepts.* Trends Biochem Sci, 2013. 38(8): p. 378-85.
3. Ross, C.A. and S.J. Tabrizi, *Huntington's disease: from molecular pathogenesis to clinical treatment.* Lancet Neurol, 2011. 10(1): p. 83-98.
4. HD_Collaborative_Research_Group, *A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group.* Cell, 1993. 72(6): p. 971-83.
5. Sah, D.W. and N. Aronin, *Oligonucleotide therapeutic approaches for Huntington disease.* J Clin Invest, 2011. 121(2): p. 500-7.
6. Cha, J.H., et al., *Altered brain neurotransmitter receptors in transgenic mice expressing a portion of an abnormal human huntington disease gene.* Proc Natl Acad Sci U S A, 1998. 95(11): p. 6480-5.
7. Davies, S.W., et al., *Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation.* Cell, 1997. 90(3): p. 537-48.
8. DiFiglia, M., et al., *Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain.* Science, 1997. 277(5334): p. 1990-3.
9. Orr, A.L., et al., *N-terminal mutant huntingtin associates with mitochondria and impairs mitochondrial trafficking.* J Neurosci, 2008. 28(11): p. 2783-92.
10. Ravikumar, B., et al., *Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease.* Nat Genet, 2004. 36(6): p. 585-95.
11. Trushina, E., et al., *Mutant huntingtin impairs axonal trafficking in mammalian neurons in vivo and in vitro.* Mol Cell Biol, 2004. 24(18): p. 8195-209.
12. Banez-Coronel, M., et al., *A pathogenic mechanism in Huntington's disease involves small CAG-repeated RNAs with neurotoxic activity.* PLoS Genet, 2012. 8(2): p. e1002481.
13. de Mezer, M., et al., *Mutant CAG repeats of Huntingtin transcript fold into hairpins, form nuclear foci and are targets for RNA interference.* Nucleic Acids Res, 2011. 39(9): p. 3852-63.
14. Nalavade, R., et al., *Mechanisms of RNA-induced toxicity in CAG repeat disorders.* Cell Death Dis, 2013. 4: p. e752.
15. Ross, C.A., *Polyglutamine pathogenesis: emergence of unifying mechanisms for Huntington's disease and related disorders.* Neuron, 2002. 35(5): p. 819-22.
16. Harper, S.Q., et al., *RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model.* Proc Natl Acad Sci U S A, 2005. 102(16): p. 5820-5.
17. Kordasiewicz, H.B., et al., *Sustained therapeutic reversal of Huntington's disease by transient repression of huntingtin synthesis.* Neuron, 2012. 74(6): p. 1031-44.
18. Krauss, S., et al., *Translation of HTT mRNA with expanded CAG repeats is regulated by the MID1-PP2A protein complex.* Nat Commun, 2013. 4: p. 1511.
19. Trockenbacher, A., et al., *MID1, mutated in Opitz syndrome, encodes an ubiquitin ligase that targets phosphatase 2A for degradation.* Nat Genet, 2001. 29(3): p. 287-94.
20. Aranda-Orgilles, B., et al., *The Opitz syndrome gene product MID1 assembles a microtubule-associated ribonucleoprotein complex.* Hum Genet, 2008. 123(2): p. 163-76.
21. Lea, W.A. and A. Simeonov, *Fluorescence polarization assays in small molecule screening.* Expert Opin Drug Discov, 2011. 6(1): p. 17-32.
22. Machleidt, T., et al., *NanoBRET--A Novel BRET Platform for the Analysis of Protein-Protein Interactions.* ACS Chem Biol, 2015. 10(8): p. 1797-804.

23. **Stanek, L.M., et al.,** ***Silencing mutant huntingtin by adeno-associated virus-mediated RNA interference ameliorates disease manifestations in the YAC128 mouse model of Huntington's disease.*** **Hum Gene Ther, 2014. 25(5): p. 461-74.**

24. **DiFiglia, M., et al.,** ***Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits.*** **Proc Natl Acad Sci U S A, 2007. 104(43): p. 17204-9.**

25. **Sengupta, S., T.R. Peterson, and D.M. Sabatini,** ***Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress.*** **Mol Cell, 2010. 40(2): p. 310-22.**

26. **Schweiger, S., et al.,** ***Resveratrol induces dephosphorylation of Tau by interfering with the MID1-PP2A complex.*** **Sci Rep, 2017. 7(1): p. 13753.**

Figure Legends

Figure 1. Peptides derived from α4 inhibit the interaction with MID1.

(A) Domain structure of MID1 and α4 proteins. The regions covered by the MID1 BBox1 and BBox1/2 constructs is shown, as is the region of α4 from which the peptides were derived. (B) Binding of α4 peptide by MID1 BBox proteins. Indicated amounts of MID1 BBox1 (open symbols) or BBox1/2 (closed symbols) were added to a fixed amount (10 nM) of Alexa647-labelled α4 peptide (GSK'454A) and the change in fluorescent polarisation (FP) was measured. (C) Inhibition of FP signal by unlabelled α4 peptide (GSK'037A). Indicated amounts of GSK'037A were used to inhibit the FP binding signal at BBox1 (open circles) and BBox2 proteins (closed squares). Peptides containing sequences for HIV-TAT prior to the WT α4 sequence (GSK'364, open triangles) or a point mutant (GSK'365 (Y271A), inverted triangles) were also tested using the BBox1 construct. Data presented are from a single representative experiment. A complete listing of data is provided in supplementary Table1. (D) Full length versions of MID1 and α4 proteins were expressed in HEK cells using the Nano-BRET system. Under basal conditions the interaction between the proteins generated a BRET signal. Addition of GSK'364 to the cell cultures resulted in a rapid decrease in the measured BRET signal while GSK'365 had no effect. Data shown are mean ± SEM of 3 independent experiments for GSK'364 and 2 independent experiments for GSK'365.

Figure 2. Characterisation of Huntingtin expression in long-term CGN cultures.

(A) Ca^{2+} imaging of DIV16 CGN with no nutrient replenishment showed that neurons (23 out of 25) were responsive to excitatory stimuli. Grey traces represent recording from individual cells and the red trace is the mean 340/380 ratio of 25 cells plotted in 1 min bins. (B) Wild-type and mutant HTT bands can be separated on a large format midi gel. Samples are of $Hdh^{Q7/Q7}$ (WT), $Hdh^{Q111/Q7}$ (HET) and $Hdh^{Q111/Q111}$ (HOM) mouse brains. (Ci and Cii) One-way ANOVA showed an effect of days for mHTT, $F_{(6,14)}$=19.19; wtHTT, $F_{(6,14)}$=36.38; and (Ci and

Ciii) pS6, $F_{(6,14)}$=47.27 (all p<0.0001). Post-hoc Dunnett's test confirmed a significant decrease in protein levels (all p<0.05). HTT and pS6 expressions were decreased in cultured CGN with no replenishment of fresh nutrients, even though GAPDH expression was normal

Figure 3. Nutrient replenishment is important for continued HTT expression in long-term primary CGN culture.

(A i and ii) when refreshment of nutrients occurred every 2-3 days, HTT expression was consistent throughout 21 days in vitro (DIV). One-way ANOVA confirmed there was no effect of days (mHTT, $F_{(5,12)}$=1.20; wtHTT, $F_{(5,12)}$=0.40). (A i and iii) pS6 expression fluctuated with nutrient replenishment. # increase in pS6 expression coincided with recent media change. There is significantly more pS6 expression on DIV3 compared to the rest of the time in culture (One-way ANOVA $F_{(5,12)}$=15.76; post-hoc Dunnett's test p<0.05). ((B) Phalloidin staining of actin filaments demonstrated a healthy morphology of CGN culture on DIV15.

Figure 4. Treatment with GSK'364A peptide reduced HTT expression in primary CGN.

(A) α4 peptide treatment for 24 hr significantly reduced HTT expression and S6 phosphorylation (i, ii, iii). Expression of MAP2, GFAP and the house-keeping proteins GAPDH, was not affected by GSK'364A or GSK'365A treatment. Bars represent mean ± SEM. (A ii) One-way ANOVA showed significant effect of peptides mHTT: $F_{(2,15)}$=11.31; wtHTT: $F_{(2,15)}$=8.51; p<0.01. Post-hoc Dunnett's test confirmed a significant effect of GSK'364A on wtHTT, and GSK'364A and GSK'365A on mHTT (p<0.05). (A iii) Significant effect of peptides on pS6 (one-way ANOVA $F_{(2,15)}$=11.38, post-hoc Dunnett's test p<0.05). (B i and ii) α4 peptide treatment for 24 hr had no effect on PP2A in cultured CGN. (B iii) S6

phosphorylation was reduced. Note that tubulin was used as a loading control since PP2A and GAPDH are of similar molecular weight.

Interest Statement:

Conflicts of interest: none

Contributors:

O.M., R.B., C.C, A.A., R.B., C.P., E.J., A.B., K.G. and S.K. undertook work included in the manuscript.

J.L., R.L., S.S. and I.U. designed and oversaw experiments.

O.M., C.C., R.B., S.S. and I.U. prepared and reviewed the manuscript.

All authors have approved the final article.

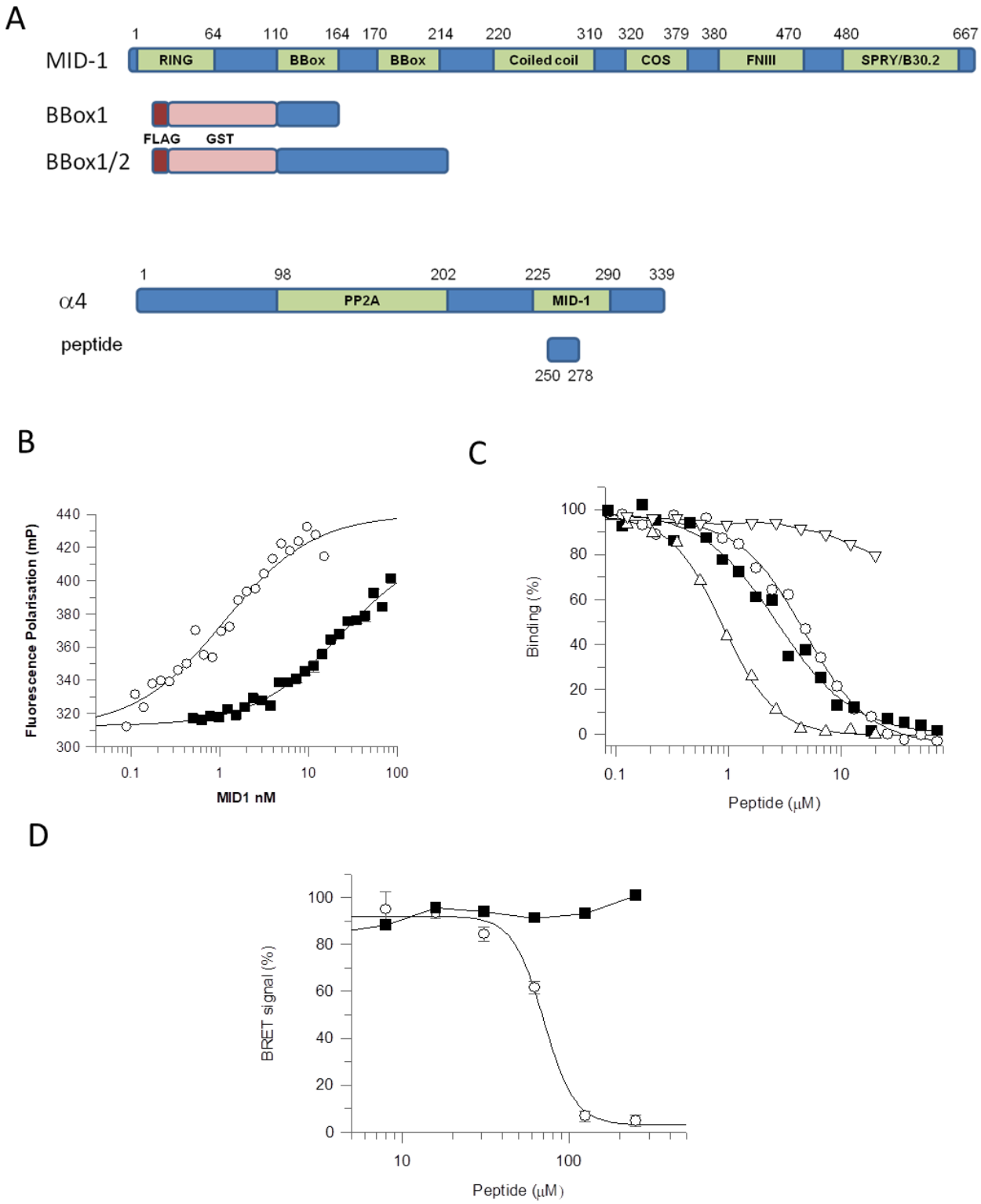

Figure 1. Peptides derived from α4 inhibit the interaction with MID1.

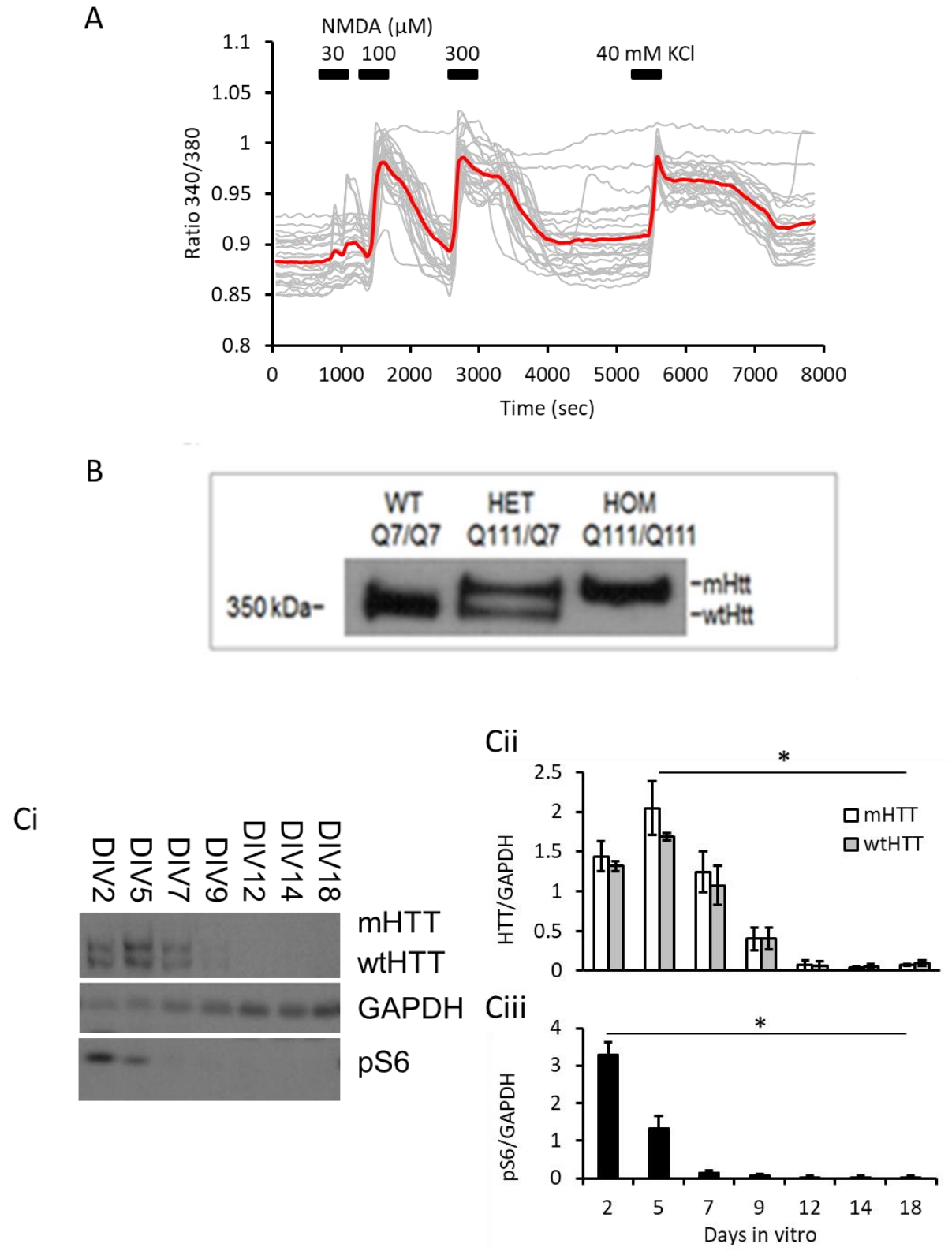

Figure 2. Characterisation of Huntingtin expression in long-term CGN cultures.

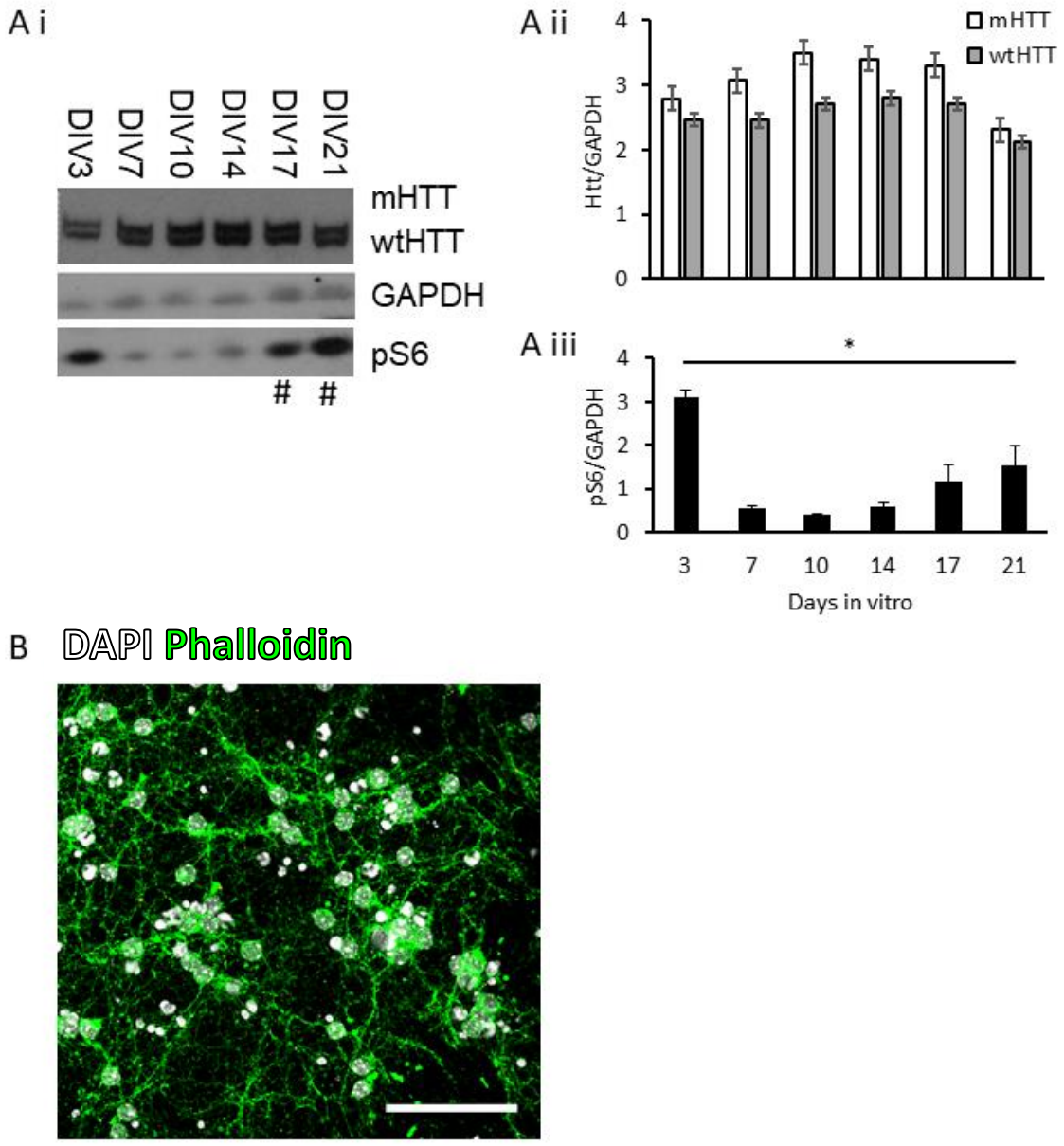

Figure 3. Nutrient replenishment is important for continued HTT expression in long-term primary CGN culture.

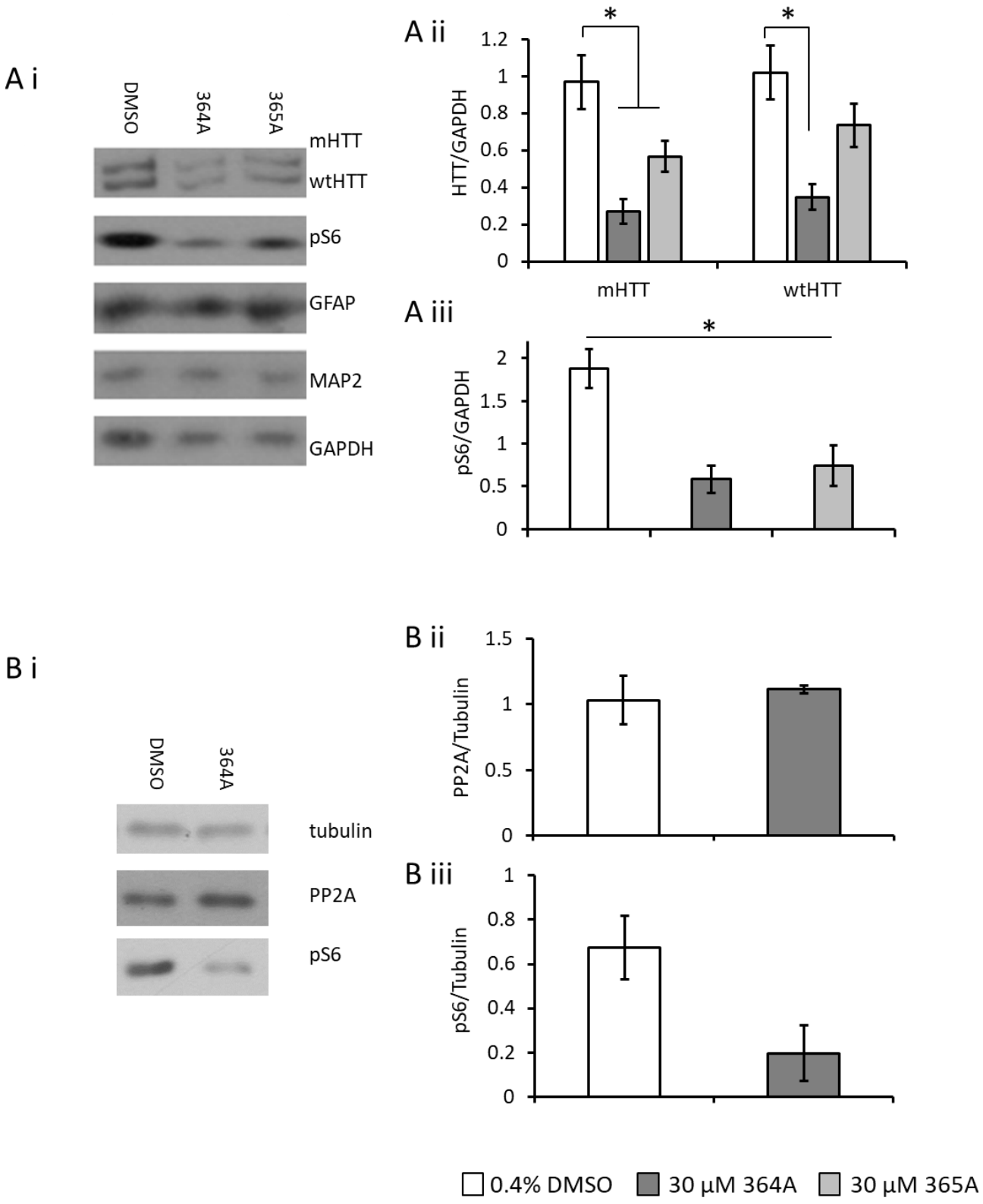

Figure 4. Treatment with GSK'364A peptide reduced HTT expression in primary CGN.